Participation of minerals in various functionalized aqueous solution or "Function Water", in exertion of detoxification effect on organotin intoxicated *Euglena gracilis*

Mari Ohta, Kaoru Nakamura*, Kozo Takama and Tetsuya Suzuki[1]

(*Laboratory of Food Wholesomeness, Department of Marine Bioresources Chemistry, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041, Japan; phone: + 81-138-40-5564; e-mail, ted @pop.fish.hokudai.ac.jp; * Institute of Chemical Research, Kyoto University, Uji, Kyoto, Japan*)

Abstract

Processing water with various mineral materials, electrostatic field, magnetic field gives functionalized aqueous solution, so-called "Function Water" in Japan. Series of research in our laboratory has revealed that restoration of morphology and motility of Tributyltin-chloride (TBTCl) intoxicated protozoa, *Euglena gracilis* should be due to Ca and Mg ions in the processed aqueous solution. In order to examine whether the detoxification effect could be observed commonly in the so-called "Functional Materials", we compared the extent of detoxification effect of various commercially available materials by evaluating restoration of motility of TBTCl-intoxicated *Euglena gracilis*. Materials examined in the present study were reduced-state mineral concentrate, glassy surface ceramic, and zeolite materials partly substituting Na with Fe, Mg, Mn, Zn, and Cu separately. Processed aqueous solutions were prepared by dissolving mineral concentrate in different concentrations, or immersing the materials for a period of time. TBTCl-intoxicated *Euglena gracilis* cells were separately washed with the processed aqueous solutions, then incubated in the solutions for up to 3 hrs. The restoration of morphology and motility was evaluated by observing the motile cell number under the video-microscope. Remarkable restoration was observed in the 250 folds diluted reduced mineral concentrate and solutions treated with zeolites encaging Fe, Mn, and Zn, respectively. However, those solutions did not show any restoration effect when they were treated with a chelator, Chelex-100. These results suggest that minerals should take part as the critical role in detoxification of TBTCl in Euglena cells. It may also be considered that glassy surface ceramic treatment should be explained by other mechanisms.

---

1) To whom all the correspondence should be mailed.

Introduction

"Function Water" or "Physiologically functional aqua-solution" is generic term given to water species that exhibit biological function superior to the control aqua-solution system prepared with distilled water[1]. However, the name is not authorized scientific term yet. So-called "Function Water" species are prepared by treatment with high-electric field loading, magnetic field loading[2], contact with ceramics, piezoelectric element[3], mineral concentrate etc. The authors have studied "Function Water" from the standpoint of scientific evaluation. Previously, we reported a water species which was prepared by high-electric field loading in the presence of charcoal (wood ceramics) produced at above 1000℃ heating definitely restored the morphology and cell motility of TBTCl-intoxicated *Euglena gracilis* Z[4]. During the course of studies to reveal what made it effective on the restoration of cell morphology and motility, we showed data suggesting that its effect should not be due to water cluster size but minerals (possibly Ca)[5]. Further comparative studies on the effect of restoration of Euglena cell morphology and motility after TBTCl intoxication showed that water species prepared by alternate current electric field loading with wood ceramics (Charged water) and that prepared by immersing alumina-iron ceramic were apparently effective[6].

Furthermore, TBTCl has been known to affect membrane function and ATPases[7,8] which have been regarded as the energy source of morphological restoration and cell motility. In the previous report[8], the authors revealed that the restoration of cell motility by "Function Water" species was closely related with ATPase activity in connection with Ca and/or Mg.

Even though, so-called "Function Water" and "Functional Materials" on the market still have been left as "mysterious" or "miraculous" without clear scientific explanation. In the present study, we tried to find whether the effect of physiologically functional materials would have common characteristic; *i.e.*, detoxification effect could be recognized and their effect could be explained by specific minerals.

Materials and Methods

"Function Water" material examined

As found in the previous preliminary experiment, water species prepared by the mineral concentrate exhibited as high as restoration effect of Al-Fe ceramics treated water, the mineral concentrate was chosen to examine its effect on restoration of TBTCl-intoxicated *E. gracilis* Z. "Chemically Reduced Mineral Concentrate" was kind gift from Dr. H. Hayakawa, Hayakawa Institute (Ibaragi, Japan). According to Dr. Hayakawa's explanation, the mineral concentrate was prepared from sea water after removing NaCl.

Since the basic structure of Al-Fe ceramics is similar to that of zeolite, the basic chemical formula of which is alminosilicate, we tried to examine the restoration effect of zeolites in which Fe, Mg, Mn, or Cu were encaged separately at the share of 2.3-12.4% by substituting Na in zeolite type A. Materials used

Table 1. "Functional Materials" examined

Materials	Treatment for use	Remarks
Hayakawa Mineral Concentrate ®	diluted with water at the concentration of 100～1000 folds dilution	
Shinei Ceramics	water was circulated through cylinder filled with ceramics (ca. 500 pieces) in 6cm i.d. x 46cm long for 15min.	Glassy surface ceramics manufactured at above 1000℃.
Mineral encaged Zeolites		Substitute (%)
Fe^{2+}-encaged	suspended in pure water for 15min by 5% w/w ratio, then filtered through 0.45 μm Millipore ® filter.	9.14
Mn^{2+}-encaged	same as above	5.71
Cu^{2+}-encaged	same as above	7.24
Zn^{2+}-encaged	same as above	12.41
Mg^{2+}-encaged	same as above	2.80

Substituent (%) represents the proportions of individual mineral replaced with Na present in the original zeolite.

in the present study are listed in Table 1. Zeolite encaged with minerals were prepared and kindly supplied by Dr. Kurihara at Shinagawa Fuel Corp. For the treatment of water, ultrapure water was prepared by filtration through hollow fiber tube. Prior to its use the treated water was filtered through membrane filter (0.2 μm; Millipore Corp.). In order to examine the participation of minerals the water species prepared by treatment with materials listed in Table 1 were used in the assay test using *Euglena gracilis* Z. As the control water species, ultrapure water was prepared by doubly distilled, then filtered through hollow fiber, and finally filtered through Chelex-100 column to remove minerals. In case of EGTA treatment EGTA was added to the water at the final concentration of 250mM to chelate minerals such as Ca and/or Mg.

Euglena cell strain

Euglena gracilis Z grown on the Koren-Hutner medium[9] at 28℃ under illumination (2800 lx) with 12 hours intervals for 7 days was used for the TBTCl intoxication and subsequent restoration experiments.

Intoxication of Euglena cells

After incubating *Euglena gracilis* Z for 7 days, cells were harvested and provided for TBTCl intoxication as described in our previous paper[10].

Evaluation of restoration of cell morphology and motility

Restoration of cell morphology and motility were examined by video microscopy (ARGUS 100, Hama-

matsu Photonics, Hamamatsu, Japan) as reported earlier[4,5]. Namely, Euglena cells were intoxicated by contacting to 50 μM TBTCl at final concentration for 1 min. After washing with the processed water species shown in Table 1 for 3 times, Euglena cells in cyst form (spherical form) were suspended with the processed water species in an Eppendorf tube, and incubated for 180 min at 28℃ under illumination (2800 lx). At regular time interval, cells were examined microscopically under inverted microscope (Olympus IMT-2) equipped with video image analyzer (ARGUS-100, Hamamatsu Photonics, Hamamatsu, Japan). The number of motile cell (or spindle form) was counted on the video images, then restoration efficiency was calculated dividing motile cell numbers by total cell numbers in at least different 10 images.

The restoration efficiency was compared with time, and the data were evaluated by Q-test and student's t-test.

Analysis of elements in processed water species

Composition of elements in the processed water species was analyzed by inductively coupled plasma atomic emission spectrometry using Japan Jarrell-Ash model 96-953 according to the analytical condition of Sugiyama et al.[11].

Results and Discussion

1) Evaluation of cell motility recovery potential of functional materials

Restoration of motility of TBTCl-intoxicated Euglena cells by incubating with processed water species was compared (Table 2). Data presented in Table 2 are motile cell proportion

Table 2. Comparison of restoration of motility of TBTCl-intoxicated *Euglena gracilis* Z by "Function Water" species

"Function Water" species	Motile cell % after 3hrs incubation
Control†	16.8 ± 4.02
Hayakawa Mineral Concentrate	
250 diluted soln.	52.1 ± 9.50**
100 diluted soln.	15.9 ± 5.85^N
Shinei Ceramics	32.4 ± 7.51*
Fe^{2+}-encaged zeolite	57.2 ± 8.89**
Zn^{2+}-encaged zeolite	55.8 ± 8.23**
Mn^{2+}-encaged zeolite	48.1 ± 7.49**
Mg^{2+}-encaged zeolite	21.5 ± 4.08*
Cu^{2+}-encaged zeolite	11.3 ± 4.30^N

Motility percent was calculated dividing motile cell numbers by the total cell numbers at 3 hr after starting incubation with water species processed with "Functional Materials". Data presented are average values ± S.D. calculated from different 10 microscopic fields, or n = 10. ** : $p < 0.001$, * : $p < 0.005$, ^{N} : no significant difference to control data.

†Control datum was obtained from incubating cells in the ultrapure water prepared by the procedure described in the text. Symbols with the different superscript mean significant difference by the student's t-test.

observed after incubation for 3 hrs.

As shown in Table 2, significant restoration was recognized in Hayakawa Institute's chemically reduced mineral concentrate at 250-fold dilution. Zeolites encaging Fe, Zn, and Mn also showed significant restoration effect. Restoration effect of glassy surface ceramic was not so high as those above though, some extent of restoration effect was recognized in comparison with that of the control. On the other hand, the mineral concentrate at 100-fold dilution showed no effect, and Cu-encaged zeolite treated water was rather lower than the control.

2) Examination on the participation of trace minerals in "Function Water" on the restoration of cell motility of Euglena cells.

As shown in the Table 2, chemically reduced mineral concentrates exhibited significant restoration effect at the concentration of 250-fold dilution. Since similar results were recognized for specific minerals substituted zeolites (Table 2), we examined whether their restoration effect could be blocked if those water species were treated separately with chelators, 250mM EGTA and Chelex-100, prior to incubation of TBTCl-intoxicated Euglena cells. Fig. 1 and 2 show how chelators affected restoration of motility of Euglena cells by water species produced by "Function Water" material. It is obvious that the effect of chemically reduced mineral concentrate at 250-fold dilution was obstructed by the treatment with chelator. On the other hand, the effect of ceramics with glassy surface was not affected by chelator. The effect of

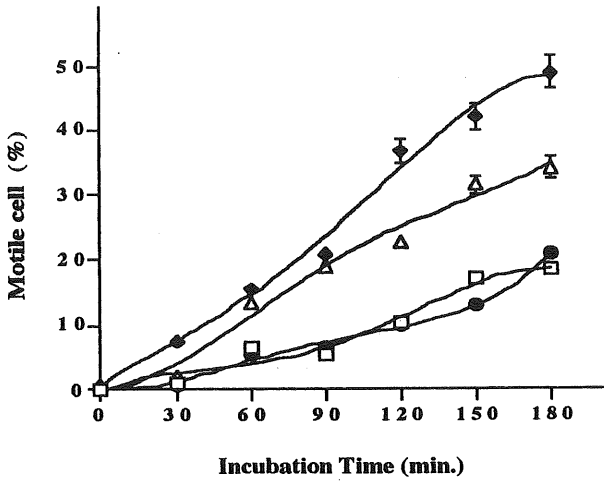

Fig. 1. Recovery of cell motility of TBTCl-intoxicated Euglena cell by incubation with mineral concentrate (Hayakawa Institute)

□ Control : Incubated in the ultrapure water

◆ Incubated in the mineral concentrate : 250 dil.

● +Chelex : Water species containting mineral concentrate (250dil.), then treated with Chelex-100

△ + EGTA : Water species containing mineral concentrate (250dil.), then chelated by 250 mM EGTA

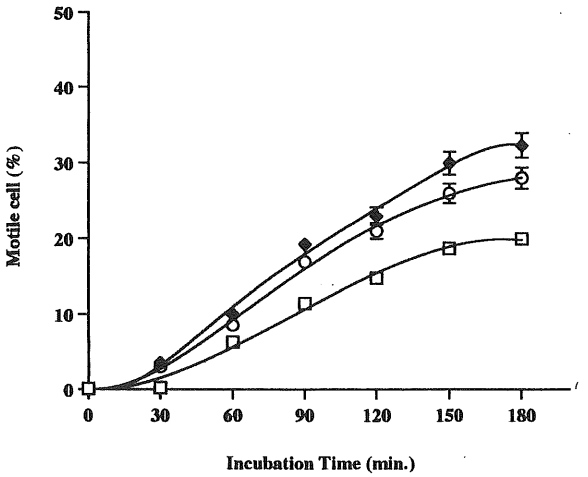

Fig. 2. Recovery of cell motility of TBTCl-intoxicated Euglena cell in glassy surface ceramics (Shinei Ceramic)[6] (30 min) treated water.

□ Incubated in the control : Ultrapure water ; TBTCl-intoxicated Euglena cells were incubated in the ultrapure water.

◆ Incubated in the water species processed by glassy surface ceramics for 30 min.

○ + Chelex-100 : glassy surface ceramics (30 min) treated water species was processed by Chelex-100 prior to incubation.

Fe-encaged zeolite was blocked by Chelex-100 treatment, but it was not affected by treatment with 250mM EGTA (data not shown).

3) Analysis of mineral composition of "Functional Materials" by ICP

As described above, minerals seem to participate in the restoration of cell motility, mineral composition of mineral concentrate was analyzed by ICP. In Fig. 3, mineral composition of mineral concentrate and mineral composition after treatment with Chelex-100 is also presented. It is clear that Ca and Mg are abundant in the mineral concentrate. Whereas the mineral concentrate treated with Chelex-100 was extremely low in Ca and Mg. Therefore, Ca and Mg are thought to play important role in detoxification of TBTCl in Euglena cells as common minerals as recognized in Al-Fe based ceramics[6].

The inhibitory effect of TBTCl on Euglena cell is partly due to inhibition of ATPases[10], and Ca and Mg have been known to participate in enzyme activities located in membrane fraction of mitochondria and other membrane fractions. On the mechanism of heavy metals such as organotin and their toxicity there are many research papers by Selwyn and co-workers[12].

It is also clear from the data presented in our papers that Ca and Mg play important roles in restoring cell motility[6,10]. However, excess amount of those minerals rather inhibited the restoration of cell motility and morphology. It means that inhibition of the enzyme activity is due to excess concentration of Ca and Mg.

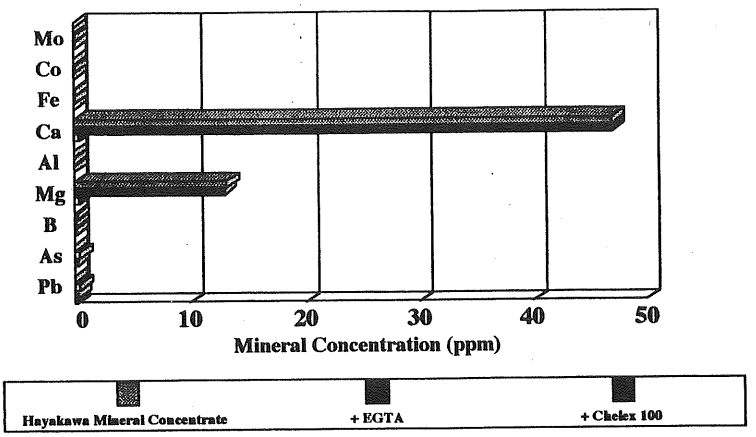

Fig. 3. Major elements in the water species treated with functional materials
Major elements in the mineral concentrate (Hayakawa Institute) at 250-fold dilution with ultrapure water was measured by ICP.
+ EGTA : mineral concentrate at 250-fold dilution with ultrapure water was treated with 250mM EGTA before subjecting to ICP analysis.
+ Chelex-100 : mineral concentrate at 250-fold dilution solution was filtered through column packed with Chlelex-100, then subjected to ICP analysis.

The restoration effect of glassy surface ceramics should be explained by the different mechanism made for mineral concentrate and zeolite. It may be due to mineral and water clathrate structure formed by piezoelectric effect as shown in tourmaline[10]. By the X-ray microanalysis and X-ray diffraction analysis the glassy surface ceramic has been revealed to be composed of SiO_2, Al_2O_3 and FeO, but its surface is glass-like structure, or amorphous structure. The detailed mechanism of its effect should be cleared by further investigation.

Although the detailed study on the mechanism of zeolites has not yet been finished, zeolite-encaged with different types of metals may exhibit catalytic action as biotransformation enzymes[13], energy metabolism relating enzymes as well as adsorbent or act as functional water processor. Further study on the effect of metal-encaged zeolite will be reported in other paper[14].

Acknowledgments

Authors are indebted to Dr. Y. Kurihara, Shinanen Fuel Corp., Shinanen Zeomic Division, Nagoya, Japan for his kind preparation of zeolites. Thanks are due to Dr. H. Hayakawa, the Hayakawa Institute, Ibaragi, Japan for his kind supply of Hayakawa Mineral Concentrate and Mr. T. Imanishi, Shinei Ceramics Corp. for his kind supply of glassy ceramic. X-ray microanalysis and X-ray diffraction were made by Dr. I. Shimono, Hokkaido Prefectural Institute of Technology, Hakodate. Authors wish to express their special thanks to Miss. S. Mito, Institute for Chemical Research, Kyoto University, Uji, Japan for her

analysis of mineral composition on ICP.

References

1) Iwamoto, M. (1991) Shokuhin to Kaihatsu (Food and Development), 26(7) : 2-12.

2) Watanabe, S. (1991) Shokuhin to Kaihatsu (Food and Development), 26(7) : 14-20.

3) Matsuoka, T. and M. Iwamoto (1991) Nippon Shokuhin Kogyo Gakkaishi 38 : 422-424.

4) Suzuki, T., N. Takahashi, K. Fukunaga, M. Okamura, T. Tanaka, and K. Tahama (1993) Environm. Toxicol. Water Qual. 8 : 207-222.

5) Suzuki, T., K. Fukunaga, K. Takama, M. Okamura, A. Ohno, and T. Tanaka (1992) Trace Nutrients Res., 9 : 181-187.

6) Suzuki, T., H. Doi, I. Shimono, and K. Takama (1995) Trace Nutrients Res., 12 : 171-176.

7) Zucker R. M., K. H. Elstein, R. E. Easterling, H. P. Ting-Beall, J. W. Allis, and E. J. Massaro (1988) Toxicol. & Appl. Pharmacol. 96 : 393-403.

8) Pinkney, A. E., D. A. Wright, M. A. Jepson, and D. W. Towle (1989) Comp. Biochem. Physiol. 92C : 125-129.

9) L. E. Koren, and S. H. Hutner (1967) J. Protozool., 14, (suupl.) : 17.

10) T. Suzuki, R. Ikeuchi, and K. Takama (1996) Trace Nutrients Res., 13 : 105-110.

11) Sugiyama, M. O, Fujino, S. Kihara, and M. Matsui (1986) Anal. Chim. Acta, 181 : 159-168.

12) Teo, T. S. P. Thiyagarajah, M. J. Lee, M. K. Selwyn (1993) Biochem. Med. & Metabol. 50 (3) : 358-362.

13) Parton R. F. (1994) Nature 370 : 541.

14) Ohta M., K. Nakamura, K. Takama, and T. Suzuki Analyst, (in press).